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Sent: Wednesday, May 09, 2001 2:32 PM
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ANALYSIS OF FACTORS CONTROLLING PRIMARY GERM LAYER FORMATION AND EARLY HEMATOPOIESIS USING EMBRYONIC STEM CELL IN VITRO DIFFERENTIATION

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Differentiation and subsequent development are intricately interwoven processes operating as an integrated whole to form the organism. As an approach to examine these interactions in early mammalian development, we used embryonic stem (ES) cell in vitro differentiation. ES cells can, depending upon the environment differentiated to neuroectoderm, mesoderm and hematopoietic cells. We developed a serum-free, chemically defined medium (CDM) in which ES cells survive and differentiate. In CDM, in the absence of exogenous factors, ES cells form neuroectoderm, upregulating the early neural marker Pax-6. This is consistent with the view that neuroectoderm development can represent a default state, where the absence or sequestration of mesoderm inducing factors *permits* neuroectoderm formation. In contrast, if CDM is supplemented with bone morphogenetic protein (BMP) 2 or 4 a process resembling primitive streak formation, least at the molecular level occurs, with the formation of mesoderm and subsequently endothelial and hematopoietic cells. If used with care, ES cell in vitro differentiation can act as a guide in understanding the environment which controls early differentiation events in mammals.

INTRODUCTION

The developmental organization of an organism from the apparently simple egg to a fully functional animal is the product of a long evolutionary history. This history has led to the formation of highly complex integrated systems with a "logic" which is often difficult to unravel. We have began to examine the processes involved in the earliest stages of germ layer formation in mammals using the relatively accessible model of mouse embryonic stem (ES) cell in vitro differentiation. In mouse, primitive ectoderm begins to form the primary germ layers, i.e. mesoderm, neuroectoderm and endoderm, at 6.0 to 6.5 days post coitum (p.c.). By day ~8.0 p.c. the first somites are visible and hematopoietic precursor cells are detectable in the extraembryonic mesoderm. Slightly later, hematopoietic cells are detectable in the embryo proper in the vicinity of the developing aorta, gonad, mesonephros (the AGM region, (1)). These data are defining the regions where hematopoietic cells are first detected, but there is little information regarding the actual factor/s which mediate their development from mesoderm (or even those responsible for the formation of mesoderm). In part, this is due to the difficulty of manipulating the mouse embryo, which at this stage is only 100–200 μm in length. Additionally, there is also a paucity of understandable model systems which bear some resemblance to the early stages of mammalian development.

ES cell in vitro differentiation does offer a potentially experimentally amenable system with which to gain an understanding of the control of early mammalian development. ES cells are derived and are similar to the inner cell mass of the 3.5 day blastocyst. They have a normal karyotype and can give rise to the complete development of the mouse (2, 3). Further, they can be maintained as cell lines in a primitive/undifferentiated state in the presence of leukemia inhibitory factor (LIF) and fetal bovine serum (FBS). In the absence of LIF but with FBS, ES cells "spontaneously" differentiate to ectoderm, endoderm, mesoderm and many mesodermal derivatives, including hematopoietic cells (4–6).

ES cell differentiation in vitro can be compared with model systems based on microdissected regions of the amphibian Xenopus laevis blastula – the animal cap assay. Using these assays, it has been shown in amphibian that defined polypeptide factors can influence primary germ layer formation, including the development of hematopoietic cells (see review and references therein (7)). However, "man is not a frog" and Xenopus's early development is quite different from mammals; for example the Xenopus egg is heavily prepatterned by differential placement of RNAs and proteins, this degree of prepatterning is not seen in

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mammals. ES cell in vitro differentiation experiments are conceptually similar to those using the Xenopus animal cap, however a major difference lies in the type of medium used for the culture of these cells. One of the advantages of using Xenopus animals cap assays is that the cells are cultured in a simple salt solution, to this neutral background specific growth factors can be added, and their effects monitored. This is not the case with ES cell experiments, where the medium is routinely supplemented with 10–20% serum. Serum contains many ill defined substances which have to a large extent confounded the systematic defining of those factors involved in early mammalian development.

In previous work we developed an efficient method which *allows* ES cells, as embryoid bodies (EBs), to differentiate. This used conventional tissue culture medium with FBS, plus the presence of monothioglycerol (a reducing agent). Although this showed that ES cells have the potential to be used as a model system to study early development, it also clearly indicated that in the presence of FBS, ES cell differentiation in vitro was in general refractory to exogenous added growth factors (6). Worse, it also confirmed the well established idea that the batch of FBS used in these experiments is the single most important component in obtaining good ES cell in vitro differentiation.

RESULTS AND DISCUSSION

To circumvent these problems we developed a serum free, chemically defined tissue culture medium, CDM. This medium is composed of commercially available, chemical defined reagents and supports ES cell growth and differentiation (8). In the presence of FBS and the absence of LIF, FBS mediates mesoderm development. Under serum free conditions in CDM, ES cells rapidly lose their ES phenotype but do not form mesoderm (8), instead neuroectodermal rapidly develops (as indicated by Pax-6 expression (manuscript submitted)). This is consistent with neuroectoderm development being a favored differentiation pathway in the absence of mesoderm inducing substances (9, 10).

The TGF-β family has been implicated in the formation of mesoderm in Xenopus (see review (7)) and very recently in mouse (11). As such we examined the effects of a number of TGF-βs on ES cell differentiation in CDM. We observed that activin A, bone morphogenetic proteins 2 or 4 (BMP -2, -4) in CDM mediated mesoderm development. Further the type of mesoderm which developed was influenced by both the concentration and the nature of the factor. Activin A mediated the formation of dorsoanterior-like mesoderm, whilst BMP -2 or -4 facilitated the formation of posterior-ventral mesoderm (8). Of particular interest here, was that both BMP-2 and 4 also mediated the formation of hematopoietic precursors cells and the expression of hematopoietic lineage markers, including GATA-1, GATA-2, embryonic and adult globins, Ikaros and the endothelial marker Flk-1 plus its ligand, vascular endothelial growth factor (VEGF). Hematopoietic differentiation was further increased by the addition of VEGF, possibly indicative of a link between endothelial cells and hematopoiesis (i.e. possibly the haemangioblast).

Although these data clearly indicated that ES/EBs can respond to both activin A and BMP-2 or 4 in vitro, it does not prove that these factors are active in mesoderm induction and hematopoiesis in vivo. Members of the superfamily belonging to particular classes (e.g. the BMPs, Inhibins etc.) are very similar in the "pro-domain" region of the active protein and a degree of receptor promiscuity has been observed (12), and review (13). Thus apparent redundancy can be observed when TGF-β function is examined in vivo (e.g. via knockout experiments in mice) and in vitro, where specific effects are seen on tissues/cells which do not correspond to the in vivo situation. In order to begin to address this question we examined the expression of both activin A and BMP-4 during mesoderm formation in vivo. Activin βA RNA is present in decidua tissue at 6 to 7 days of development (6, 14). Further, BMP-4 RNA is detectable in day 6.5 mouse egg cylinder and in undifferentiated ES cells, although the expression level in both is quite variable (8, 15). Thus these factors are present and could be involved in mesoderm and for BMP-4, also hematopoietic development.

The idea that two factors are responsible for mesoderm development is almost certainly a gross over simplification of the situation. It is becoming obvious as more data is acquired, that mesoderm formation involves a coalition of many factors, with no single factor being the prime instigator of *all* mesoderm (or hematopoietic development?). This is clearly shown, for example, with BMP-4 knockout mice, where anterior mesoderm still forms in the null mutant embryos (11). This suggests that although BMP-4 is crucial for the formation of posterior structures, it is probably only one of a group of factors which act in a coordinated manner to either induce and/or pattern mesoderm.

CONCLUSION

The studies outlined in this report clearly show that mouse ES cell in vitro differentiation can be influenced

by the addition of specific growth factors in the absence of FBS. Using this system we have evidence that BMP-4, or a very similar molecule is a player in the formation of mesoderm and subsequent hematopoietic cell development. However, an individual factor is only one part of an interwoven network of controls which function at the level of the whole organism. The high degree of developmental assurance/compensation observed reflects the evolutionary nature of life; such systems are difficult to analyze and understand. Using ES cell in vitro differentiation is not a simplification of the mechanisms involved, it does, however offer the possibility to break these networks down into experimentally amenable units. If *used with care*, this model system will give us deeper insights into the processes which control early mammalian development.

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